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Long-term in situ Thiol Monitoring in Living Cells Using Bioorthogonal Chemistry

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Contents

1. Synthetic materials and methods

Materials

Tetraacetylated N-azidoacetyl-d-mannosamine (Ac4ManNAz) was purchased from Thermo Fisher Scientific Inc. (USA), and used as recommended.

Ethyl 2-oxo-1-cyclooctanecarboxylate (97%), 4-amino-1,8-naphthalic anhydride (95%), 2-hydroxyethyl disulfide (90%), D-biotin (99%), L-glutathione reduced (GSH, 98%), L-cysteine (Cys, 97%), DL-homocysteine (Hcy, 95%), Thioredoxin human (Trx, 90%) were purchased from Sigma-Aldrich Co. LLC. (USA), used as received.

Selectfluor® fluorinating reagent (Selectfluor, 95%), *N*-phenyl-bis(trifluoromethanesulfonimide) (Tf_2NPh, 99%), potassium bis(trimethylsilyl)amide (KHMDS, 1 M in THF), lithium hydroxide monohydrate (98%), triphosgene (99%) were purchased from Aladdin Industrial Inc. (China), and used as received

Tetrahydrofuran (99.5%, extra dry over molecular sieve, stabilized) was purchased from J&K Scientific Ltd. (China), used as received.

N,N-Diisopropylethylamine (DIEA, 99%), Bis(dimethylamino)methylene]- 1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU, 99%), 1-Hydroxy-7-azabenzotriazole (HOAt, 99.5%) were purchased from GL Biochem (Shanghai) Corporation. Ltd. (China), used as received.

Ethylamine (60%-70% aqueous solution), triethylamine, acetonitrile, ethyl acetate, dichloromethane, methanol, *n*-hexane, diethyl ether, dimethyl sulfoxide (DMSO), N,N-dimethylformamide (DMF), hydrochloric acid (HCl), sodium sulfate anhydrous (Na_2SO_4) were AR grade and purchased from Shanghai Chemical Co. (China).

Instrumentation

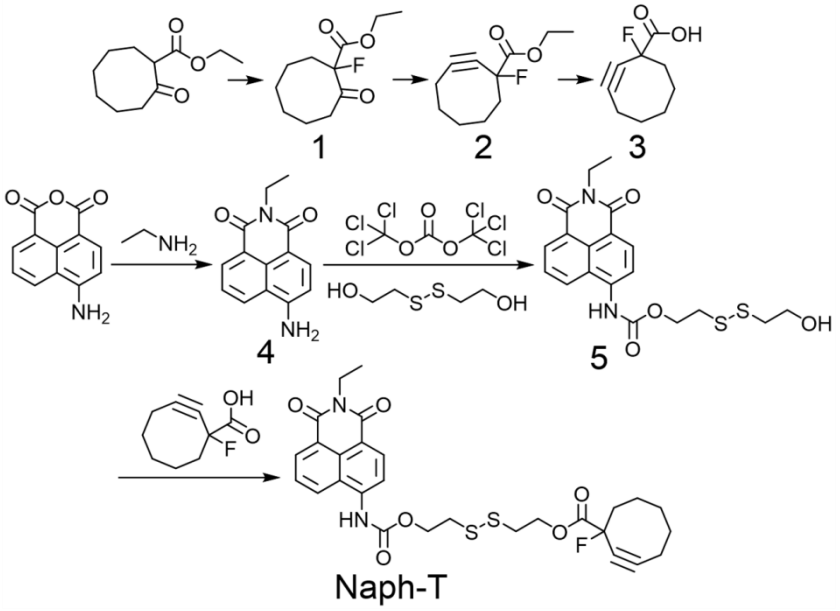
NMR

^{1}H and ^{13}C NMR spectra were recorded on a Varian Unity 300 MHz spectrometer. All chemical shifts are reported in ppm using either residual proton signals of the deuterated solvent or TMS as an internal reference. All deuterated solvents were provided by Sigma-Aldrich Co. LLC.

HPLC/MS

ESI mass and spectral analyses were carried out using a HPLC/MS-6120 instruments (Agilent Technologies 6120 Quadrupole LC/MS Series).

Synthesis of Naph-T



Scheme S1. Synthetic routes to Naph-T

The compound 3 was synthesized according to the reports of Timmerman with little

alteration.[S1]

Synthesis of compound **1**

10 g methyl-2-oxocyclooctane-1-carboxylate in were solved in dry acetonitrile cooled to 0 °C and 28.9 g Selectfluor was added. The resulting mixture was stirred at 55 °C for 8 h. After cooling to room temperature the reaction was quenched with water (200 mL) and extracted with ethyl acetate (3 x 100 mL). The combined organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo to yield clear oil which was then dissolved in CH_2Cl_2 and filtered through a plug of silica gel to give **1** as yellowish liquid (8.2 g, 75%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 4.24 (q, 2H), 2.73-2.47(m, 3H), 2.31-2.20(m, 1H), 2.07-1.85(m, 2H), 1.74-1.66(m, 3H), 1.51-1.39(m, 3H), 1.31-1.25(m, 3H); ^{13}C-NMR (75MHz, CDCl3) δ 280.68(d, CO), 167.17(d, CO), 97.67(d, Cq), 62.48(CH2), 38.84(CH2), 33.27(d, CH2), 27.72(CH2), 26.46(CH2), 24.43(CH2), 21.44(d, CH2), 13.94(d, CH3).

Synthesis of compound **2**

23 mL KHMDS (1 M in THF) was added dropwise to a stirred solution of 2 g **1** in THF (125 mL) at -78 °C and maintained for 30 min. After which 3.89 g Tf_2NPh in THF (25 mL) was added slowly to the reaction mixture and stirred at -78 °C for an hour. Then the reaction mixture was allowed to warm to room temperature and stirred overnight. Methanol was then added to quench the reaction and reaction mixture was concentrated under vacuum. The crude residue was purified by flash column chromatography on silica gel using 2-5% ethyl acetate in hexane as eluent to afford **2** as yellow liquid (0.6 g, 32%).

^{1}H-NMR (300 MHz, $CDCl_3$) δ 4.26 (q, 2H), 2.38-2.24(m, 4H), 2.05-1.82(m, 4H), 1.76-1.69(m, 1H), 1.50-1.43(m, 1H), 1.32(t, 3H); ^{13}C-NMR (75MHz, $CDCl_3$) δ 168.26(d, CO), 108.15(d, Cq), 92.54(d, Cq), 86.70(d, Cq), 62.02(t, CH2), 46.17(d, CH2), 33.68(CH2), 28.92(CH2), 25.33(CH2), 20.35(CH2), 14.02(d, CH2).

Synthesis of compound **3**

0.65 g **2** and 0.17 g LiOH were combined in ~10 mL of 50% aqueous/methanol, and stirred at 50 °C for 10 min. Then cooling to room temperature and stirred for an additional 2 h. The reaction mixture was cooled to 0 °C, diluted with water, and acidified to pH ~2 with dilute aq. HCl solution. The mixture was extracted with ethyl acetate (3 x 10 mL) dried over anhydrous Na_2SO_4, filtered, and concentrated to afford **3** as a yellow oil (0.15 g, 88%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 8.86(bs, 1H), 2.42-2.29(m, 4H), 2.12-1.86(m, 4H), 1.77-1.71(m, 1H), 1.52-1.43(m, 1H); ^{13}C-NMR (75MHz, $CDCl_3$) δ 173.88(d, CO), 109.46(d, Cq), 90.42(d, Cq), 86.24(d, Cq), 46.28(d, CH2), 33.98(CH2), 29.21(CH2), 25.66(d, CH2), 20.69(d, CH2).

Synthesis of compound **4**

0.6 g 4-amino-1,8-naphthalic anhydride was dissolved in DMF (15 mL), 2 mL ethylamine (65-70% aqueous solution) were added dropwise and the reaction mixture was stirred at 100 °C overnight. The solution was cooled to room temperature and poured into ice-cold water (200 mL). The precipitate was filtered and washed with water, followed by ether, and dried over air. Yield 0.45 g, 67%. ^{1}H-NMR (300 MHz, DMSO-d6) δ

8.55(d, 1H), 8.36(d, 1H), 8.13(d, 1H), 7.60(t, 1H), 7.38(s, 2H), 6.77(d, 1H), 3.98(d, 2H), 1.12(s, 2H); ^{13}C-NMR (75MHz, DMSO-d6) δ 164.20(d, CO), 163.36(CO), 153.31(Cq), 134.52(Cq), 131.56(Cq), 130.30(Cq), 129.90(Cq), 124.59(Cq), 122.48(Cq), 120.03(Cq), 108.80(Cq), 108.30(Cq), 34.89(CH2), 13.99(CH3).

Synthesis of compound **5**

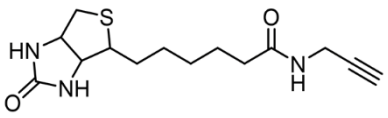
0.24 g **4** was dissolved in 30 mL dichloromethane, 0.594 g triphosgene were added and the reaction mixture was stirred at 0 °C for 20 min. After which 840 µL triethylamine was added and stirred for another 60 min. Then 1.4 mL 2-hydroxyethyl disulfide were added and stirred at room temperature for 48 h. The precipitate was filtered out and washed with 50% aqueous/methanol and dried over vacuum. Yield 0.673 g, 76%. ^{1}H-NMR (300 MHz, DMSO) δ 10.34(s, 1H), 8.67(d, 1H), 8.47(q, 2H), 8.14(d, 1H), 7.82(t, 1H), 4.46(t, 2H), 4.05(q, 2H), 3.66(t, 2H), 3.10(t, 2H), 2.86(t, 2H), 1.21(t, 3H); ^{13}C-NMR (75MHz, DMSO-d6) δ 163.95(d, CO), 163.41(CO), 154.58(CO), 141.30(Cq), 132.27(Cq), 131.54(Cq), 130.04(Cq), 129.00(Cq), 127.06(Cq), 124.70(Cq), 122.95(Cq), 119.21(Cq), 117,95(Cq), 63.73(CH2), 60.16(CH2), 41.84(CH2), 37.45(CH2), 35.37(CH2), 13.85(CH3).

Synthesis of Naph-T

52 mg **3**, 230 mg HATU, 10 mg HOAt were dissolved in 1 mL dry acetonitrile, 120 µL DIEA were added dropwise and the reaction mixture was stirred at room temperature for 1 h. Then 100 mg **5** were added and stirred at 40 °C for 24 h. After which, solvent was removed over vacuum to give crude product as brownish sold. The crude product was

purified by flash column chromatography on silica gel using 60% ethyl acetate in hexane as eluent to afford Naph-T as yellowish sold. 62 mg, 46%. ^{1}H-NMR (300 MHz, DMSO) δ 10.34(s, 1H), 8.64(d, 1H), 8.41(q, 2H), 8.12(d, 1H), 7.78(t, 1H), 4.85(t, 4H), 4.04(q, 2H), 3.13(m, 4H), 2.32-2.21(m, 4H), 1.96-1.75(m, 4H), 1.59(m, 1H), 1.38(m, 1H), 1.21(t, 4H); ^{13}C-NMR (75MHz, DMSO-d6) δ 168.17(CO), 167.80(CO), 163.58(CO), 163.29(CO), 154.49(Cq), 141.23(Cq), 132.15(Cq), 131.42(Cq), 129.88(Cq), 128.88(Cq), 126.91(Cq), 124.47(Cq), 122.83(Cq), 118.86(Cq), 117.75(Cq), 110.01(d, Cq), 93.51(Cq), 91.06(Cq), 87.42(Cq), 87.00(Cq), 64.25(CH2), 63.52(CH2), 46.69(CH2), 46.37(CH2), 37.39(CH2), 36.79(CH2), 35.33(CH2), 34.16(CH2), 29.26(CH2), 25.76(CH2), 20.43(CH2), 13.80(CH3). ESI-MS found 573.1 $[M + H]^{+}$. Purity, over 90% by HPLC.

Synthesis of biotinylated alkyne



Biotinylated alkyne is synthesized the same method we reported before.[s2]

^{1}H-NMR (DMSO-d6) d 1.31-1.39 (m, 2H), 1.51-1.59 (m, 3H), 1.64-1.70 (m, 1H), 2.14 (t, 2H, J = 7.5 Hz), 2.64 (d, 1H, J = 12.0 Hz), 2.88 (dd, 1H, J = 5.0 and 12.0 Hz), 3.13 (t, 1H, J = 2.5 Hz), 3.15-3.18 (m, 1H), 3.89 (q, 1H, J = 2.5 Hz), 4.17-4.21 (m, 1H), 4.35-4.38 (m, 1H), 6.41 (s, 1H), 6.47 (s, 1H), 8.27 (m, 1H).

2. Spectroscopic materials and methods

Fluorescence spectra were acquired by RF-5301PC spectrofluorophotometer. (SHIMADZU, Japan.) And UV/vis absorption were acquired by Lambda 35 UV/Vis

spectrophotometer. (PerkinElmer, USA.) Typically, 1 μM Naph-T was dissolved in DMSO/water mixture, particular amount of Trx, GSH, Cys, or Hcy were added, and the final concentration of DMSO in water maintain at 1% (v/v). The mixture was stored in thermostat at 37 °C for 30 min. After which the fluorescence intensity or UV/vis absorption were acquired immediately.

3. Cells culture and imaging

Dulbecco's Modified Eagle Medium (DMEM) cell culture media, fetal bovine serum (FBS), and phosphate buffered saline (PBS, pH= 7.4) were purchased from Life Technologies Corporation (USA), and used as recommended.

Trypsin-EDTA solution (0.05%, with phenol red) and Penicillin-Streptomycin solution (10,000 U/mL) were purchased from Biological Industries Israel Beit-Haemek Ltd. (Israel), and used as recommended.

PX-12 (99%), was purchased from Tocris Bioscience, R&D Systems, Inc. (USA), used as received.

Anti-Biotin antibody (HRP), was purchased from Abcom plc. (United Kingdom), used as recommended.

Human cervix adenocarcinoma (HeLa) cells and HepG2 (Hepatocellular carcinoma, human) were purchased from China Center for Type Culture Collection (Wuhan, China).

Confocal laser scanning microscope images were taken by LSM 710 NLO laser scanning microscope with two-photon excitation unit (Carl Zeiss, Germany).

Flow cytometry data were collected by a BD Biosciences multicolor flow cytometry, BD

FACSAria™ III (USA).

Western Blot image were acquired by ChemiDocTM XRS+ imaging system (Bio-Rad Laboratories, Inc., USA).

SPAAC reaction in living cells and CLSM imaging. HeLa and HepG2 cells were cultured in DMEM supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin (100 μg/mL) (DMEM medium for short). Three days before SPAAC reaction with Naph-T, the cells were placed on glass-bottomed dishes (Corning) using DMEM medium containing 40 μM Ac_4ManNAz and incubated in a humidified atmosphere containing 5% (v/v) CO_2 at 37 °C. 3 days after, the cells were washed with PBS thrice and DMEM medium containing 40 μM Naph-T were added and incubated for 30 min at 37 °C. Then the cells were washed with PBS thrice again and fresh DMEM medium were added. The CLSM images were obtained at specific time point by confocal microscope.

Treatment of HeLa cells with PX-12. After incubated with Ac_4ManNAz-containing medium for 2 days, the medium were replaced with DMEM medium containing both 40 μM Ac_4ManNAz and 10 μM PX-12. One day later, the SPAAC reaction was performed as detailed above, washed, and DMEM medium containing only 10 μM PX-12 were added.

Treatment of HeLa cells with OKA. After incubated with Ac_4ManNAz-containing medium for 2 days, the medium were replaced with DMEM medium containing both 40 μM Ac_4ManNAz and 10 nM OKA. One day later, the SPAAC reaction was performed as detailed above, washed, and DMEM medium containing only 10 nM OKA were added.

4. Western blot experiments

Cells were lysed in RIPA buffer (Beyotime, China), centrifuged, and the supernatant was collected to examine protein expression. Protein concentrations were determined using a BCA Protein Assay Kit (Beyotime, China). In total, 30 μg of protein from each sample diluted in loading buffer (Beyotime, China) was boiled for 5 min at 95 °C, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% SDS‐PAGE, Beyotime, China), and wetly transferred onto a PVDF membrane using a glycine transfer buffer for 2 h at 200 mA, 4 °C. Then the lysates were labeled with Naph-T using SPAAC reaction (40 μM Naph-T in PBS buffer, 37 °C for 30 min), and the control samples were labeled with biotinylated alkyne using CuAAC reaction according to the report of Bertozzi.[s3] For the Naph-T labeled samples, protein bands were visualized unsing ChemiDocTM XRS+ imaging system (Bio-Rad Laboratories, Inc., USA). For the control samples, non-specific reactivity was blocked using 5% bovine serum albumin (BSA) in Tris-Buffered Saline containing Tween-20 (TBST). The blots were incubated with a horseradish peroxidase-conjugated primary antibody against biotin (1:1000; Abcam, United Kingdom) overnight at 4 °C. Blots were developed using ECL reagent (Cwbio, Beijing, China) and detected with X-ray film (Kodak, Rochester, NY). Protein bands were visualized using LiDE 110 scanner (Canon, Japan).Western blot experiments were repeated at least three times to confirm the results.

5. Supplementary data

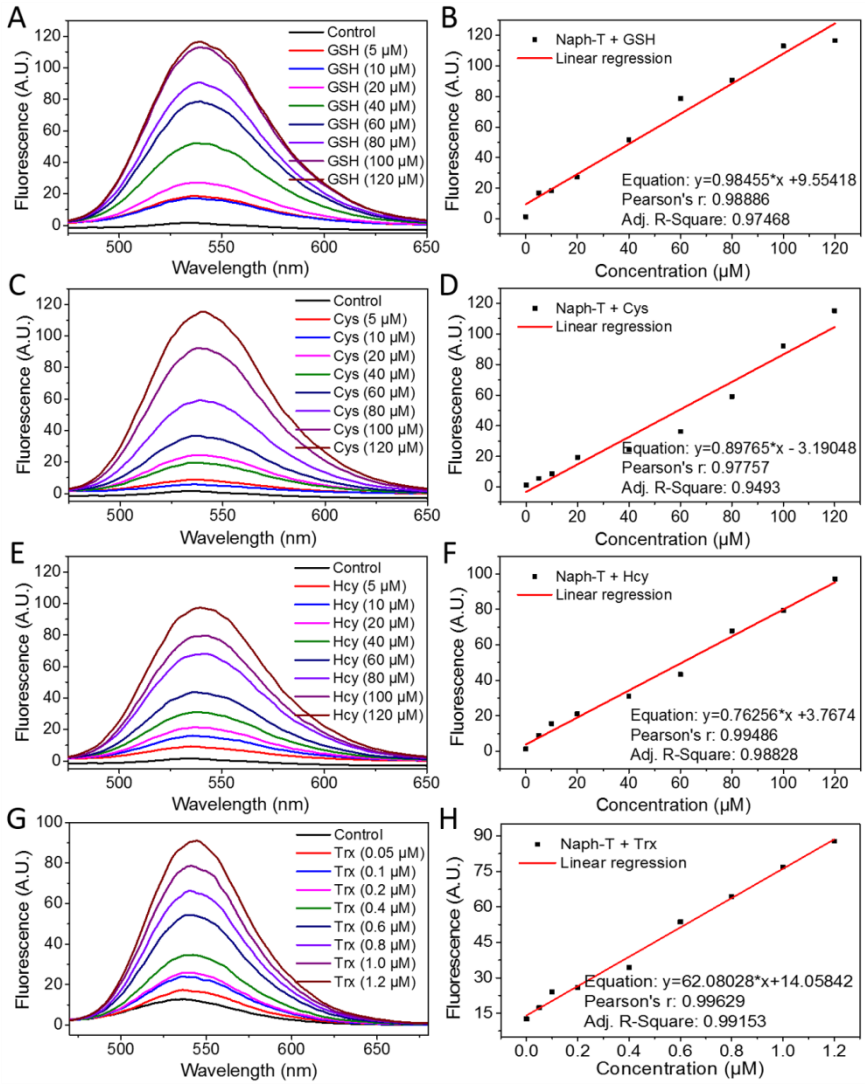


Figure S1. Fluorescence changes of Naph-T (1 μM) with increasing concentration of reduced thiol entities (GSH, Cys, Hcy, Trx) (A, C, E, G), and the linear regression between the fluorescence intensity at 450 nm and the corresponding thiol concentrations. All spectra were acquired 30 min after the addition of thiols in simulated physiological conditions (at 37 °C in PBS buffer containing 1% (v/v) DMSO, pH = 7.4)

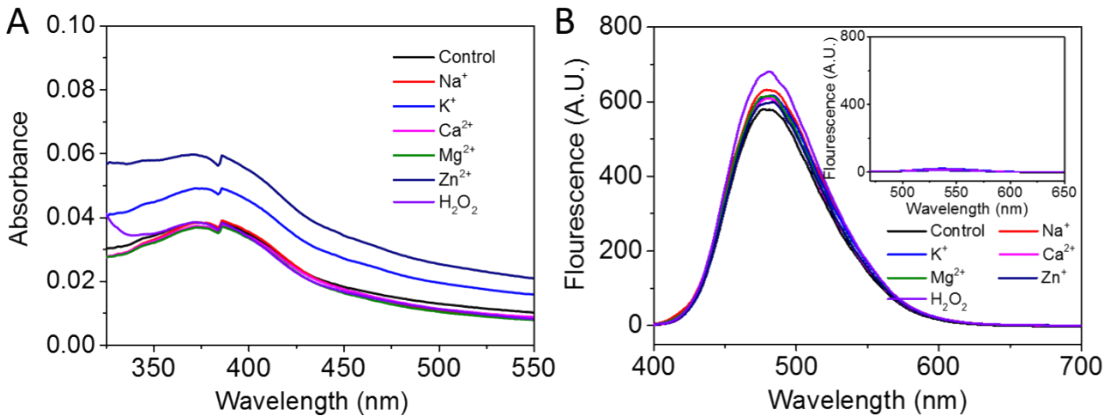
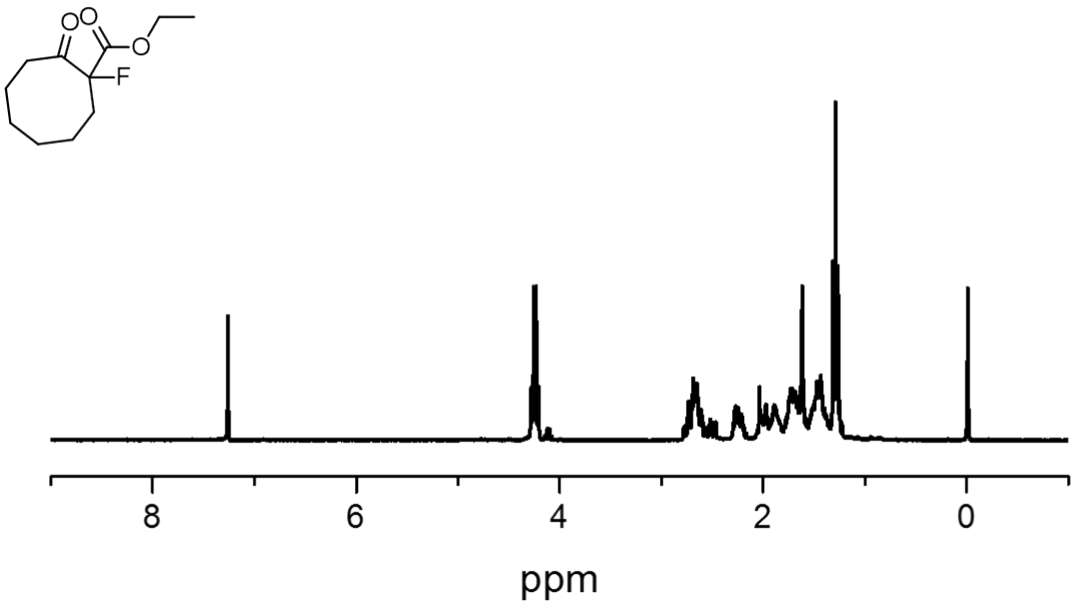
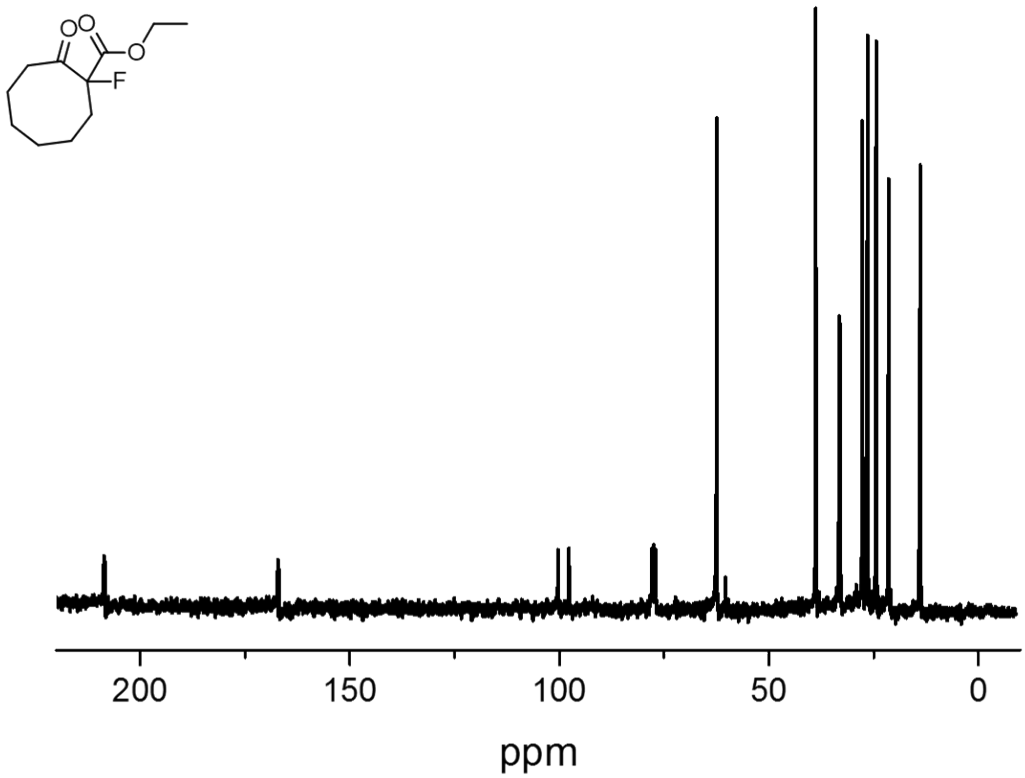


Figure S2. UV/vis absorption (A) and fluorescence spectra (B, emission at 475 nm. Inset: emission at 540nm) of Naph-T (1 μM) recorded with the presence of common cellular metal ions (100 μM), and of compound 4 (Control) (1 μM). All spectra were acquired 30 min after the addition of thiols in simulated physiological conditions (at 37 °C in PBS buffer containing 1% DMSO, pH = 7.4.). The results confirmed that, the disulfide bond in Naph-T was stable in cellular environments.

6. ^{1}H and ^{13}C NMR spectra

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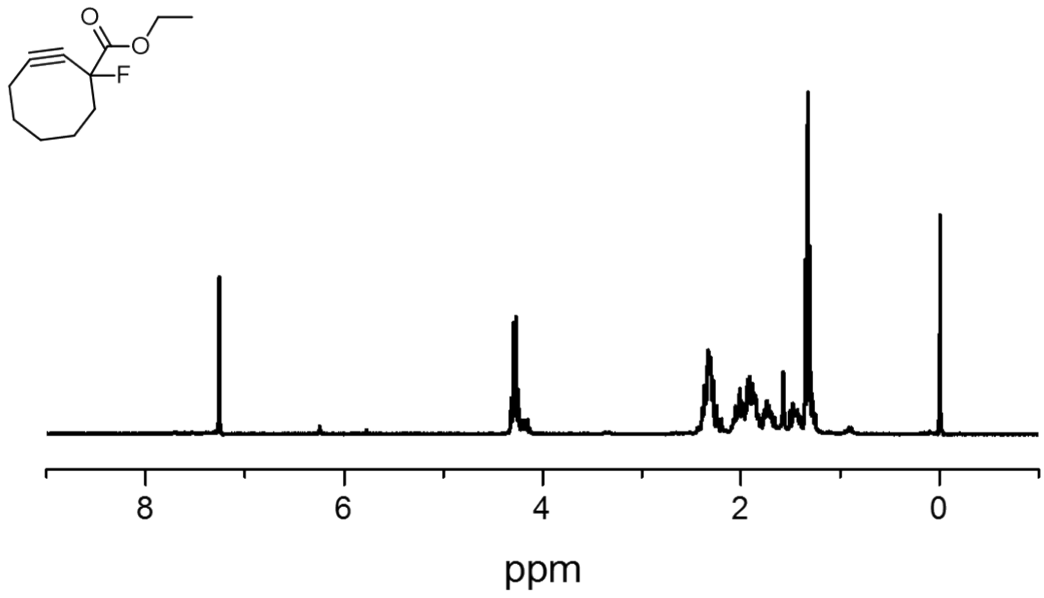
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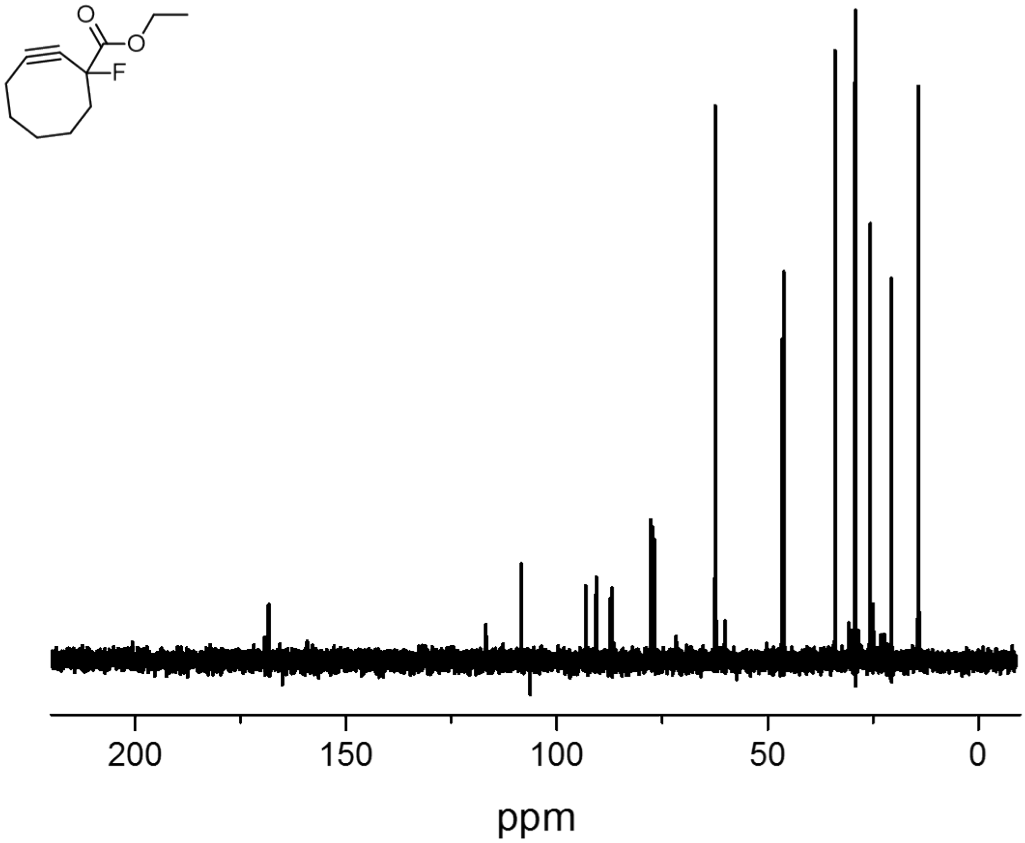
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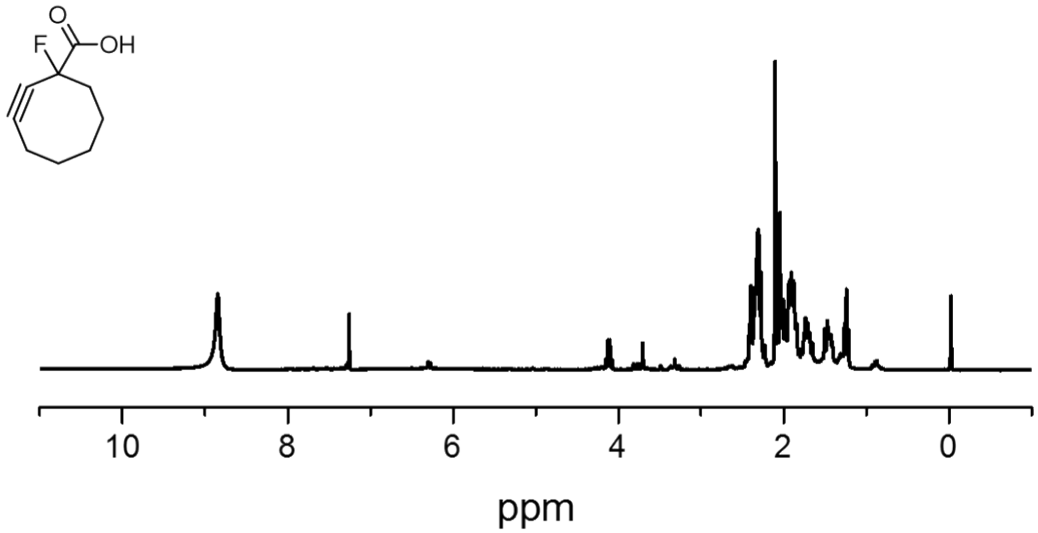
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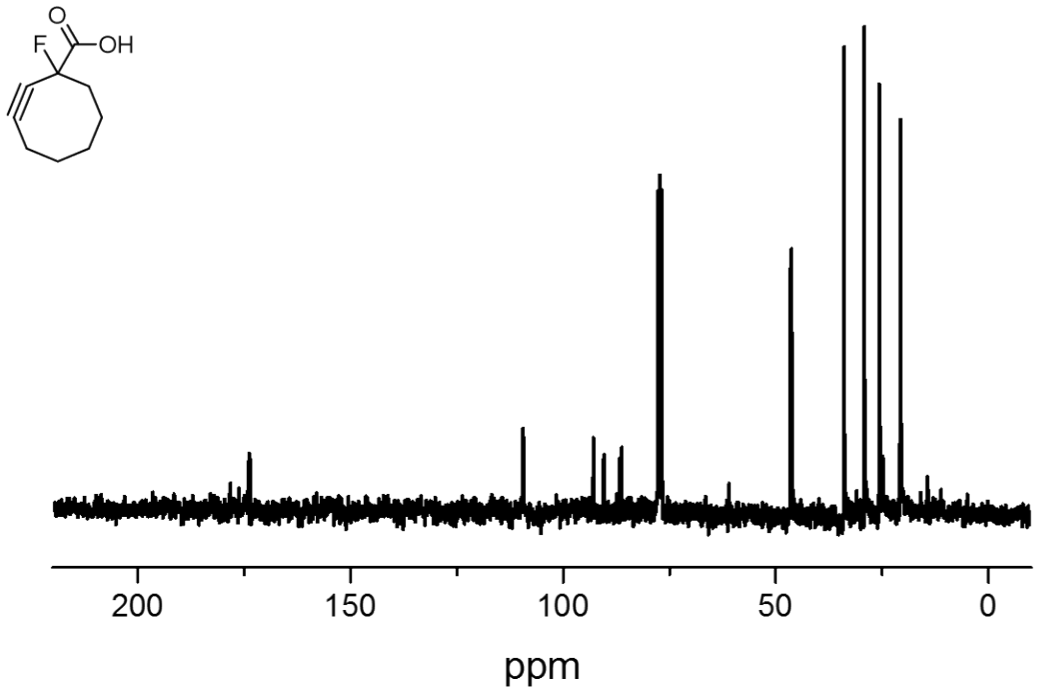
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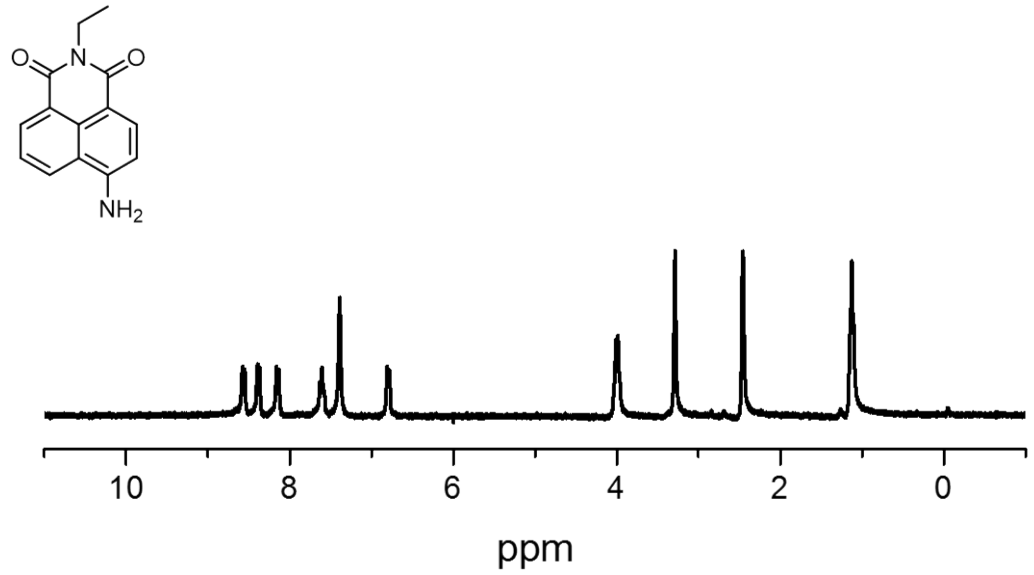
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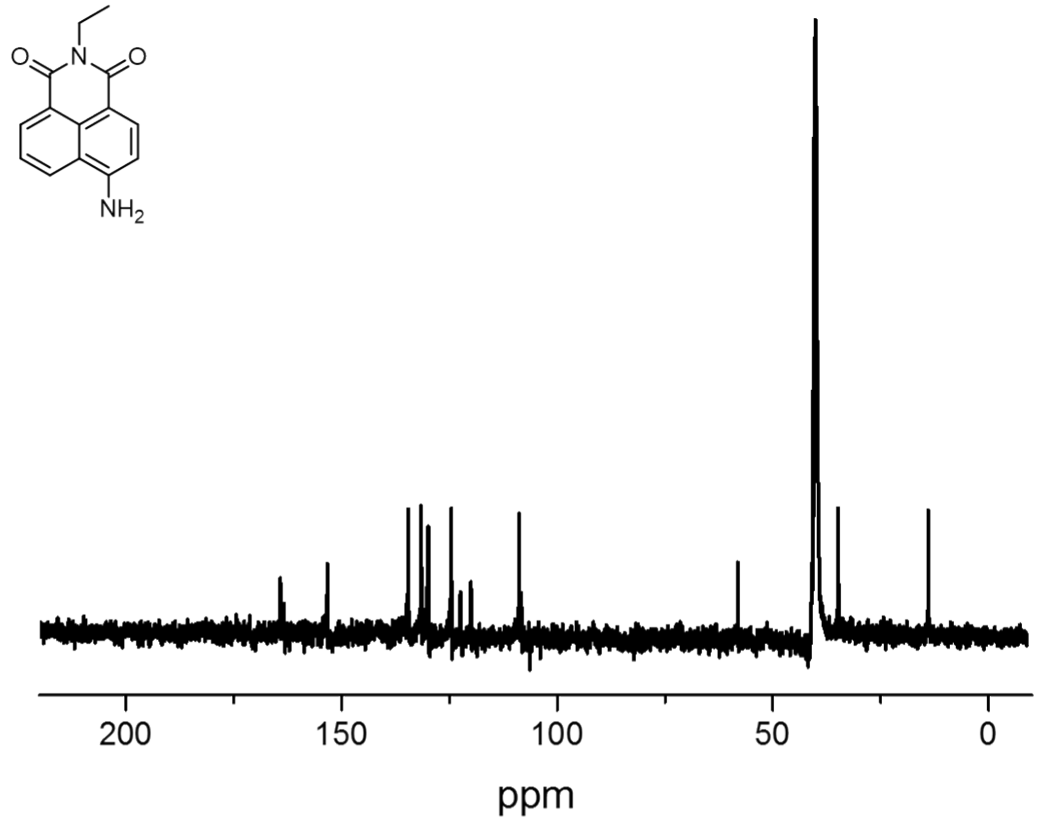
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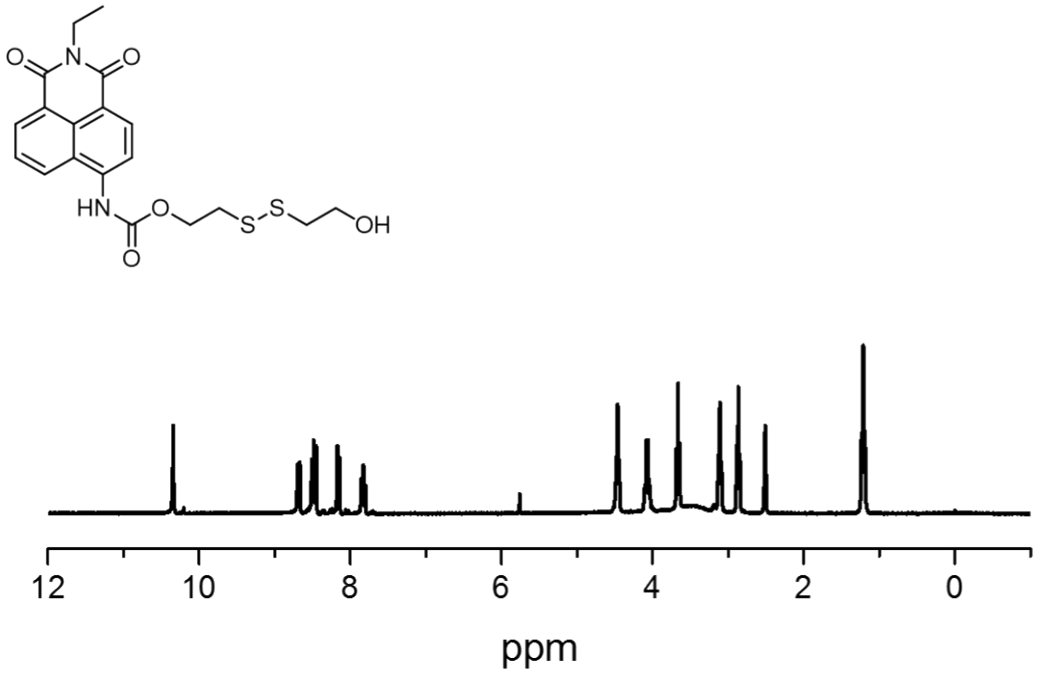
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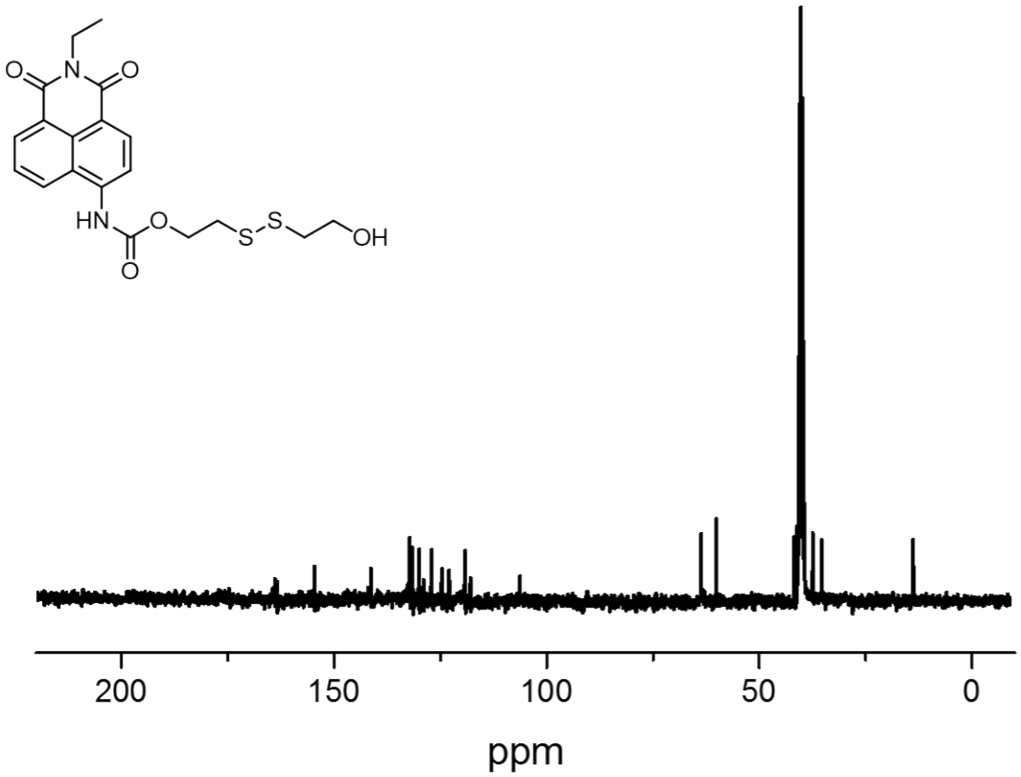
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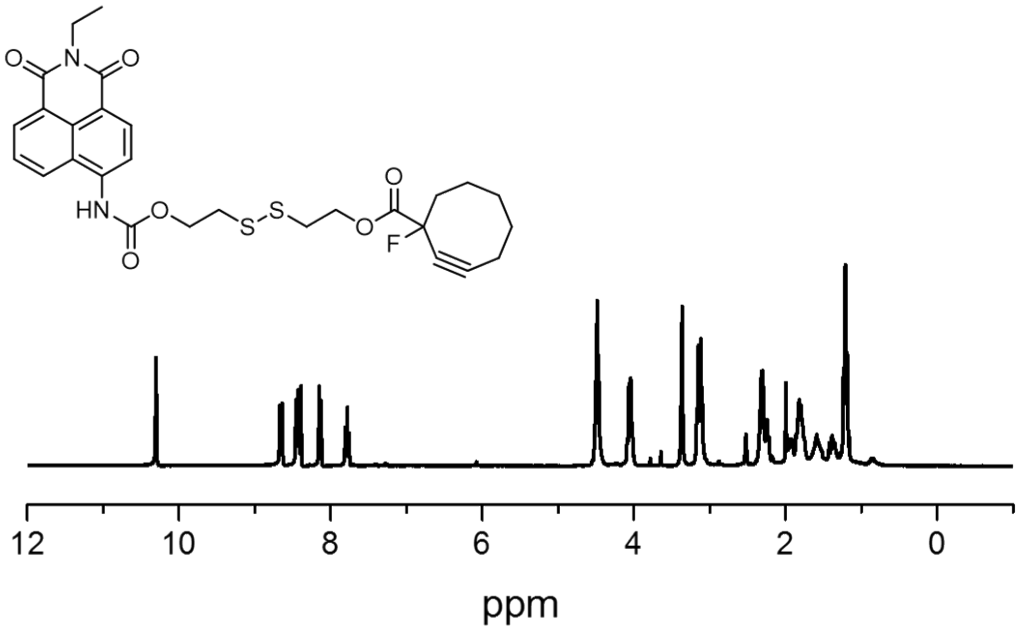
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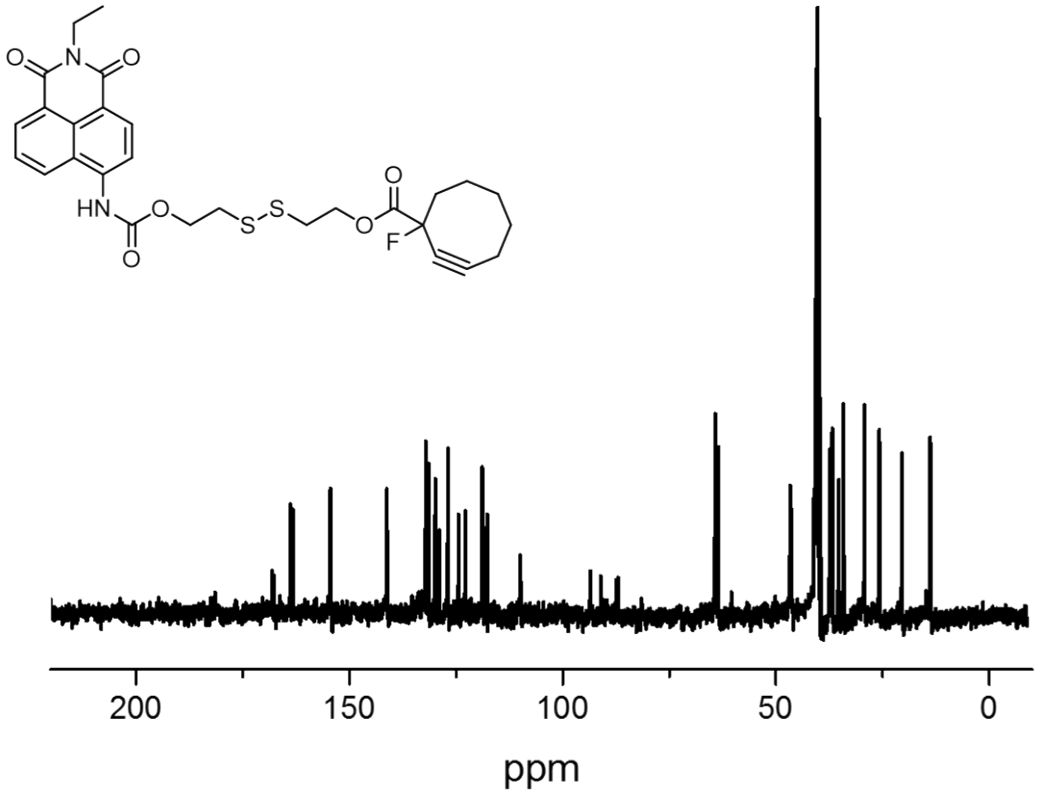

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[s2] L. Rong, L. H. Liu, S. Chen, H. Cheng, C. S. Chen, Z. Y. Li, S. Y. Qing, X. Z. Zhang, *Chem. Commun.* **2013**, *50*, 667.

[s3] P. V. Chang, X. Chen, C. Smyrniotis, A. Xenakis, T. Hu, C. R. Bertozzi, P. Wu, *Angew. Chem.* **2009**, *121*, 4090-4093; *Angew. Chem. Int. Ed.* **2009**, *48*, 4030-4033.